Heparin-Binding Epidermal Growth Factor Cleavage Mediates Zinc-Induced Epidermal Growth Factor Receptor Phosphorylation

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We have previously shown that exposure to zinc ions can activate epidermal growth factor (EGF) receptor (EGFR) signaling in murine fibroblasts and A431 cells through a mechanism involving Src kinase. While studying the effects of zinc ions in normal human bronchial epithelial cell, we uncovered evidence for an additional mechanism of Zn^{2+}-induced EGFR activation. Exposure to Zn^{2+} induced phosphorylation of EGFR at tyrosine 1068, a major autophosphorylation site, in a dose- and time-dependent fashion. This effect of Zn^{2+} on EGFR was significantly blocked with an antibody against the ligand-binding domain of the receptor. Neutralizing antibodies against EGFR ligands revealed the involvement of heparin-binding EGF (HB-EGF) in Zn^{2+}-induced EGFR phosphorylation. This observation was further supported by immunoblots showing elevated levels of HB-EGF released by Zn^{2+}-exposed cells. Zymography showed the existence of matrix metalloproteinase-3 in Zn^{2+}-challenged cells. Incubation with a specific matrix metalloproteinase-3 inhibitor suppressed Zn^{2+}-induced EGFR phosphorylation as well as HB-EGF release. Therefore, these data support an autocrine or paracrine mechanism whereby Zn^{2+} induces EGFR phosphorylation through the extracellular release of EGFR ligands, which may be mediated by metalloproteinases.

Zinc is not only an essential micronutrient involved in structural and regulatory cellular functions (1), but also a common airborne metallic contaminant that may contribute to the health effects of ambient pollution (2, 3). Our *in vitro* studies showed that exposure of human airway epithelial cells to zinc ions (Zn^{2+}) or Zn^{2+}-containing particulate matter could result in overproduction of pulmonary proinflammatory mediators (4, 5). The mechanisms underlying Zn^{2+}-related pulmonary toxicity are not well characterized. Recently, the link between the epidermal growth factor (EGF) receptor (EGFR) signaling and inflammatory pulmonary diseases has attracted much attention (6, 7).

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Abbreviations: bronchial epithelial cell basal medium, BEBM; cyclooxygenase-2, COX-2; epidermal growth factor, EGF; epidermal growth factor receptor, EGFR; heparin-binding EGF, HB-EGF; mitogen-activated protein kinases, MAPK; MAPK/ERK kinase, MEK; matrix metalloproteinase, MMP; normal human bronchial epithelial cells, NHBE; N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid, NNGH; polyacrylamide gel electrophoresis, PAGE; phosphate-buffered saline, PBS; sodium dodecyl sulfate, SDS; transforming growth factor-α, TGF-α; N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine, TPEN.

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The EGFR is the prototypal member of the ErbB family that mediates multiple cellular responses in physiologic processes as well as in pathophysiologic states (8). It has been well documented that the EGFR can be directly activated or transactivated in response to a variety of stimuli (9). In the case of direct activation, the EGFR is regulated by binding of various polypeptide ligands that contain a 6-kD domain homologous to EGF such as heparin-binding EGF (HB-EGF), transforming growth factor-α (TGF-α), amphiregulin, betacellulin, and epiregulin (10, 11). Following ligand binding, the EGFR undergoes homo- or heterodimerization and subsequent autophosphorylation of multiple tyrosine residues in the COOH-terminal cytoplasmic portion of the molecule that serve as binding sites for cytosolic signaling proteins containing Src homology 2 domains and phosphotyrosine-binding domains (12). Several *in vivo* autophosphorylation sites have been identified in the EGFR: three major (tyrosines 1068, 1148, and 1173) and two minor (tyrosines 992 and 1086) (13, 14). The binding of downstream signaling proteins to the phosphorylated EGFR initiates multiple signaling cascades, which culminate in pleiotropic biological responses such as mitogenesis, enhanced cell motility, protein secretion, and differentiation (15).

In addition to its cognate ligands, the EGFR is "transactivated" by physiologic stimuli such as G protein–coupled receptor (GPCR) ligands, cytokines, and chemokines, or nonphysiologic agents including arsenite, ultraviolet radiation, heat shock, and oxidants (8). Evidence accumulated from our recent studies suggests that exposure to exogenous zinc ions may trigger EGFR signaling in murine fibroblasts or A431 cells through a transactivation mechanism involving c-Src kinase activity (16, 17). However, recent observations suggest that the effect of Zn^{2+} may be cell type- and exposure-specific. EGFR phosphorylation and subsequent activation of the downstream mitogen-activated protein (MAP) kinase cascade has been observed in human bronchial epithelial cells exposed to Zn^{2+} (18). However, the signaling pathways that relay Zn^{2+} stimulation to EGFR activation in normal human bronchial cells are still not fully understood.

Inducible shedding of EGFR ligands recently emerged as a critical process in EGFR transactivation and subsequent cellular responses in airway epithelial cells exposed to exogenous stressors (19–24), although this mechanism was initially characterized in other cell types (9). The regulation of EGFR ligand shedding in airway epithelial cells remains to be specified. To identify the mechanism for Zn^{2+}-induced activation

of EGFR and subsequent downstream signaling in normal human bronchial epithelial (NHBE) cells, we have investigated the involvement of physiologic ligands that are known to activate EGFR and could be potentially released by Zn^{2+} treatment. In contrast to our previous observation that exposure of cells to a high dose of Zn^{2+} (16, 17) rapidly induced EGFR phosphorylation through an Src-dependent mechanism, this study shows that HB-EGF shedding exists as an additional mechanism for Zn^{2+}-induced EGFR phosphorylation in NHBE cells exposed to lower concentration of Zn^{2+} after 2 h.

Materials and Methods

Materials and Reagents

American Chemical Society–grade metal salt zinc sulfate, Triton X-100, N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), and polyacrylamide were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) supplies such as molecular mass standards and buffers were from Bio-Rad (Richmond, CA). Phospho-EGFR (Tyr-1068) antibody was obtained from Cell Signaling Technology (Beverly, MA). Goat neutralizing antibodies against EGF, HB-EGF, and TGF-α, and recombinant human HB-EGF and TGF-α, were from R&D Systems (Minneapolis, MN). Monoclonal human EGFR neutralizing antibody (clone LA1) and recombinant human EGF were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-EGFR antibody (1,005), horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti–MMP-3 (Ab-4) antibody, the EGFR tyrosine kinase inhibitor PD153035, the mitogen/ERK kinase (MEK) activity inhibitor PD98059, the Src kinase inhibitor PP2, a MMP inhibitor GM6001, a MMP-3 inhibitor N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic Acid (NNGH), a MMP-2/MMP-9 inhibitor (2R)-2-(4-biphenylylsulfonyl)amino-3-phenylpropionic Acid, and a MMP-2 inhibitor *cis*-9-octadecenoyl-N-hydroxylamide oleoyl-N-hydroxylamide were obtained from Calbiochem (La Jolla, CA). Zymogram developing buffer (10×) and 4–16% zymogram (blue casein) gel were purchased from Invitrogen Corporation (Carlsbad, CA). Purified human MMP-3 was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Chemiluminescence reagents were from Pierce Biotechnology (Rockford, IL).

Cell Culture and Exposure

Normal human bronchial epithelial cells (passages 2–3) were obtained from normal adult human volunteers by brush biopsy of the mainstem bronchi during bronchoscopy, conducted while following a protocol approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina at Chapel Hill. NHBE cells were plated on tissue culture plates coated with human collagen (Sigma) in supplemented bronchial epithelial cell basal medium (BEBM) (0.5 ng/ml human epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamycin, 50 ng/ml amphotericin-B, 52 μg/ml bovine pituitary extract, and 0.1 ng/ml retinoic acid) (bronchial epithelial growth medium [BEGM]), grown to confluence, and then cultured with BEGM deprived of epidermal growth factor for 12–16 h before challenge with zinc sulfate.

Immunoblotting

NHBE cells, untreated or pretreated with pharmacologic inhibitors or EGFR-neutralizing antibodies, were stimulated with Zn^{2+}, respectively. Cells were lysed with RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 0.1 mM vanadyl sulfate and protease inhibitors (0.5 mg/ml aprotinin, 0.5 mg/ml E-64, 0.5 mg/ml pepstatin, 0.5 mg/ml bestatin, 10 mg/ml chymostatin, and 0.1 mg/ml leupeptin). Cell lysates were subjected to SDS-PAGE, as described before (25). Proteins were transferred onto nitrocellulose membrane. Membrane was blocked with 5% nonfat milk, washed briefly, incubated with rabbit antibody against phosphorylated EGFR (Y1068) at 4°C overnight, then followed by incubation with goat anti-rabbit HRP-conjugated secondary antibody for 1 h at room temperature. Immunoblot images were detected using chemiluminescence reagents and the Gene Gynome Imaging System (Syngene, Frederick, MD).

Measurement of Released HB-EGF in Culture Medium

NHBE cells were challenged with 100 μM Zn^{2+} for 2 h. Culture media were collected and precleared by centrifugation. Released HB-EGF in the medium was trapped with heparin-Sepharose beads overnight at 4°C, respectively. After briefly washing twice with cold phosphate-buffered saline (PBS), the precipitated beads were eluted with SDS gel sample buffer. The eluted proteins were subjected to Western blotting and detected with corresponding goat anti-human neutralizing antibodies against HB-EGF. Released HB-EGF proteins were visualized with HRP-conjugated anti-goat IgG using enhanced chemiluminescence reagents.

Real-Time Reverse Transcriptase/Polymerase Chain Reaction

Total RNA from untreated or Zn^{2+}-exposed NHBE cells was isolated on cesium chloride gradients and reverse transcribed to cDNA. Oligonucleotide SYBR green primer pairs for amphiregulin, EGF, HB-EGF, TGF-α, and EGFR were designed using the primer design program Primer Express from Applied Biosystems (Foster City, CA): for amphiregulin: 5′-GTG GTG CTG TCG CTC TTG ATAC-3′ (sense), 5′-AGA GTA GGT GTC ATT GAG GTC CAAT-3′ (antisense); for EGF, 5′-TGC AGA GGG ATA CGC CCT AA-3′ (sense), 5′-CAA GAG TAC AGC CAT GAT TCC AAA-3′ (antisense); for HB-EGF, 5′-TCG CTT ATA TAC CTA TGA CCA CAC AAC-3′ (sense), 5′-CAT AAC CTC CTC TCC TAT GGT ACC TAAA-3′ (antisense); for TGF-α, 5′-CCT GGC TGT CCT TAT CAT CACA-3′ (sense), 5′-GGG CGC TGG GCT TCTC-3′ (antisense); for EGFR, 5′-GCG TCT CTT GCC GGA ATGT-3′ (sense), 5′-GGC TCA CCC TCC AGA AGC TT-3′ (antisense); for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-GAA GGT GAA GGT CGG AGT C-3′ (sense), 5′-GAA GAT GGT GAT GGG ATT TC-3′ (antisense). Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7,700 Sequence Detection System and SYBR Green Universal PCR Master Mix from Applied Biosystems. The relative abundance of mRNA of interested genes described above was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from human bronchial epithelial cells and normalized to the GAPDH mRNA levels.

Zymography

NHBE cells were treated with 100 μM Zn^{2+} for 2 h. Culture media were harvested and precleared by centrifugation. Culture medium was incubated with 10 μl of mouse monoclonal anti–MMP-3 anti-

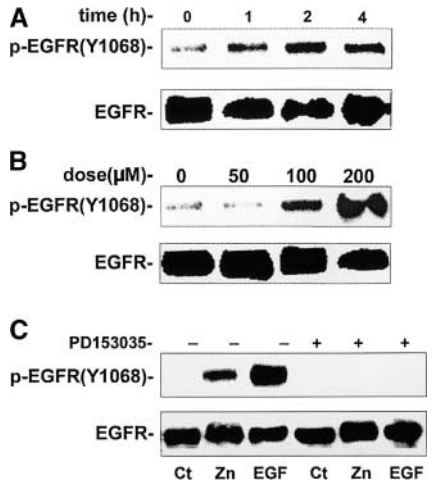

Figure 1. Zn^{2+}-induced kinase-mediated EGFR (Y1068) phosphorylation in NHBE cells. Confluent NHBE cells were starved in BEBM medium overnight before treatment with (*A*) 100 μM Zn^{2+} for 1, 2, and 4 h; or (*B*) 0, 50, 100, and 200 μM Zn^{2+} for 2 h. (*C*) Cells were pretreated with 1 μM PD153035 for 60 min before exposure to medium control (Ct) 100 μM Zn^{2+} or 100 ng/ml EGF for 2 h. NHBE cells were lysed in RIPA buffer and cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against human phospho-specific EGFR (Y1068) or total EGFR. Bands of phosphorylated EGFR or pan-EGFR proteins were detected using enhanced chemiluminescence reagents. Data shown are representative of three separate experiments.

body overnight at 4°C. Twenty microliters of Protein A-agarose were added to the medium and incubated at room temperature for 3 h. Precipitated beads were washed twice with cold PBS and mixed with an equal amount of SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.05% bromophenol blue). Media samples were applied to the prestained zymogram gel and run at 120 V constant. The gel was then incubated with zymogram renaturing buffer (2.5% Triton X-100) for 30 min at room temperature. The zymogram renaturing buffer was decanted and replaced with zymogram developing buffer to equilibrate the gel for 30 min at room temperature with gentle agitation followed by overnight incubation at 37°C in fresh zymogram developing buffer. Areas of MMP-3 proteins appeared as clear bands against a dark blue background. The purified human MMP-3 was used as a positive control.

Results

Zn^{2+} Induces EGFR Phosphorylation at Tyrosine 1068

We have previously observed that the extent of EGFR phosphorylation at specific tyrosine residues may be variable among different cell types exposed to Zn^{2+} (16, 17). Tyrosine 1068 on the C-terminus of EGFR is a major autophosphorylation site, which can transduce signals to the downstream MAPK cascade after phosphorylation (13, 14). To investigate the mechanisms responsible for Zn^{2+}-induced EGFR activation, the phosphorylation of EGFR (Y1068), was studied in NHBE cells exposed to zinc ions. Exposure to 100 μM Zn^{2+} for 1–4 h caused marked phosphorylation of EGFR (Y1068) in NHBE cells (Figure 1A). At 2 h of exposure, the effect of Zn^{2+} was evident at concentrations of 100 and 200 μM, but not 50 μM (Figure 1B). In separate experiments, EGFR phosphorylation at Y845 was detected using the same conditions, and we observed that Zn^{2+} exposure also induced EGFR phosphorylation at Y845 but the effect was much less than the phosphorylation of Y1068. Exposure of NHBE cells to 100 μM Zn^{2+} for up to 6 h did not result in significant alterations in cell viability, as assessed by assay of lactate dehydrogenase activity released into the culture medium (< 6% release). In all subsequent experiments, NHBE cells were treated with 100 μM Zn^{2+} for 2 h.

We next determined whether the phosphorylation of EGFR (Y1068) represented autophosphorylation resulting from activated EGFR tyrosine kinase activity using the EGFR kinase inhibitor PD153035 (25, 26). As shown in Figure 1C, pretreatment with 1 μM PD153035 abrogated EGFR phosphorylation induced by 100 μM Zn^{2+} or 100 ng/ml of EGF for 2 h. Thus, Zn^{2+}-induced EGFR phosphorylation in NHBE cells required receptor tyrosine kinase activity.

Zn^{2+}-Induced EGFR Phosphorylation Is Mediated by Released Receptor Ligands

We have previously shown that exposure of A431 cells to zinc ions does not directly cause EGFR dimerization as the receptor ligand EGF did (17), suggesting that Zn^{2+} exposure may lead to ligand release, thereby exerting an EGF-like effect on receptor phosphorylation. To test this hypothesis, NHBE cells were pretreated with a blocking antibody against the EGFR extracellular ligand binding domain or with normal mouse IgG control for 1 h before Zn^{2+} exposure. As expected, exposure of cells to Zn^{2+} resulted in EGFR phosphorylation in the cells pretreated with mouse IgG (Figure 2). In contrast, this effect of Zn^{2+} on EGFR phosphorylation was significantly suppressed by the receptor blocking antibody (clone LA1). The specificity of this blocking antibody was verified by its inhibitory effect on EGF-induced EGFR phosphorylation (Figure 2). These data suggested that Zn^{2+}-induced EGFR phosphorylation may be initiated via the release of EGFR ligands.

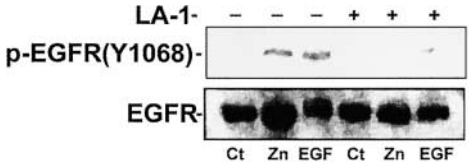

Figure 2. Zn^{2+}-induced EGFR phosphorylation (Y1068) was blocked by an antibody against extracellular ligand binding domain of the receptor. Confluent NHBE cells were starved in BEBM medium overnight and pretreated with 1 μg/ml of EGFR antibody (clone LA1) for 2 h before exposure to medium control (Ct) or 100 μM Zn^{2+} for another 2 h. Cells were lysed in RIPA buffer and cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against human phospho-specific EGFR (Y1068) or total EGFR. Bands of phosphorylated EGFR or pan-EGFR proteins were detected using enhanced chemiluminescence reagents. Data shown are representative of three separate experiments.

Zn^{2+}-Induced EGFR Phosphorylation Is Blocked by an HB-EGF Neutralizing Antibody

To examine the involvement of specific EGFR ligands in Zn^{2+}-induced receptor phosphorylation, NHBE cells were exposed to recombinant HB-EGF (Figure 3A), TGF-α (Figure 3B), or EGF (Figure 3C). Apparently, these EGFR ligands induced a dose-dependent increase in receptor phosphorylation. Furthermore, the role of autocrine or paracrine EGFR ligands were determined in Zn^{2+}-induced EGFR phosphorylation. NHBE cells were incubated with neutralizing antibodies (NA) against EGFR ligands such as EGF, HB-EGF, and TGF-α before exposure to 100 μM Zn^{2+} or 100 ng/ml EGF for 2 h. Normal goat IgG was used as a control antibody. Zn^{2+}-induced EGFR phosphorylation was not affected in the presence of the nonspecific antibody. However, anti–HB-EGF (NA) markedly reduced Zn^{2+}-induced phosphorylation of EGFR with a minimal effect on that induced by EGF (Figure 4A). In contrast, anti–TGF-α (NA) produced a partial inhibition (Figure 4B), whereas anti-EGF (NA) had no inhibitory effect (Figure 4C). These data suggested that HB-EGF played an important role in Zn^{2+}-induced EGFR phosphorylation.

Zn^{2+} Induces HB-EGF Release in NHBE Cells

To corroborate the involvement of HB-EGF in Zn^{2+}-induced EGFR phosphorylation, we next evaluated HB-EGF shedding through immunoprecipitation and immunoblotting using specific antibodies. Assays used to measure ligand appearance in the extracellular medium are typically of poor sensitivity because of the low extracellular concentration of these ligands. However, previous studies indicated that pre-incubation of cells with EGFR antibody that blocks receptor extracellular ligand binding sites increased the detection of released ligand (27). Thus, we used this strategy to enhance the detection of released HB-EGF in the medium. NHBE cells were pretreated with the blocking antibody (clone LA1) before Zn^{2+} challenge, and the culture medium was collected and analyzed for HB-EGF. As shown in Figure 5A, Zn^{2+} exposure caused a marked increase in levels of soluble HB-EGF in the medium.

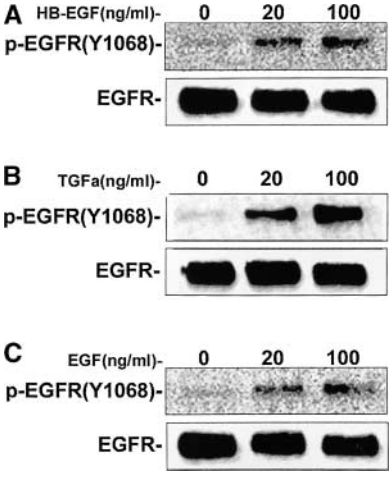

Figure 3. EGFR phosphorylation (Y1068) in NHBE cells exposed to recombinant human HB-EGF, TGF-α, or EGF. Confluent NHBE cells were starved in BEBM medium before treatment with medium control (Ct) or different concentrations of recombinant human HB-EGF (*A*), TGF-α (*B*), or EGF (*C*) for 2 h. Cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against human phospho-specific EGFR (Y1068) or total EGFR. Bands of phosphorylated EGFR or pan-EGFR proteins were detected using enhanced chemiluminescence reagents. Data shown are representative of three separate experiments.

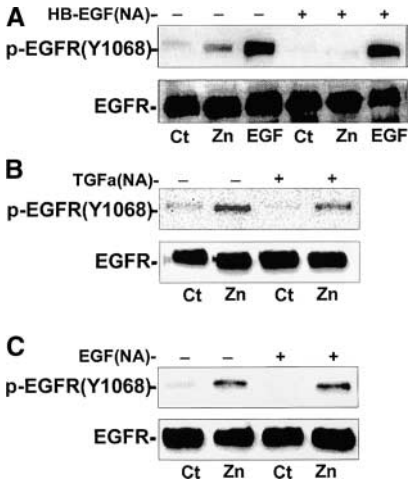

Figure 4. HB-EGF neutralizing antibody blocked Zn^{2+}-induced EGFR phosphorylation (Y1068). NHBE cells grown to confluence were starved and then pretreated for 1 h with (*A*) 20 μg/ml of HB-EGF neutralizing antibody, (*B*) 30 μg/ml of TGF-α, or (*C*) 30 μg/ml EGF neutralizing antibody. Cells were further challenged with medium control (Ct) or 100 μM Zn^{2+} or 100 ng/ml EGF for 2 h and lysed in RIPA buffer. Cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies against human phospho-specific EGFR (Y1068) or total EGFR. Bands of phosphorylated EGFR or pan-EGFR proteins were detected using enhanced chemiluminescence reagents. Data shown are representative of three separate experiments.

The effect of Zn^{2+} on mRNA expression of HB-EGF was also determined using RT-PCR. NHBE cells were treated with 100 μM Zn^{2+} for 1–4 h. HB-EGF mRNA expression was elevated by Zn^{2+} exposure (Figure 5B). In contrast, the mRNA expression of other EGFR ligands such as EGF, TGF-α, and amphiregulin or EGFR was minimally affected by Zn^{2+} stimulation under the same exposure condition (data not shown).

An MMP-3 Inhibitor Partially Blocks Zn^{2+}-Induced HB-EGF Release and EGFR Phosphorylation

Because metalloproteinases have been reported to be responsible for cleaving tethered EGFR ligand precursors (28–31), we next examined whether MMPs were involved in Zn^{2+}-induced HB-EGF release and subsequent EGFR phosphorylation. A broad-spectrum MMP inhibitor, GM6001, was used for this purpose. NHBE cells were pretreated with 40 μM GM6001 for 20 min before further stimulation with 100 μM Zn^{2+} for 2 h. As shown in Figure 6A, GM6001 abrogated Zn^{2+}-, but not EGF-, induced EGFR phosphorylation. This finding implicated MMP activation in Zn^{2+}-induced EGFR phosphorylation. To identify the specific MMP responsible for this effect, a MMP-3 inhibitor NNGH was tested under the same condition. NNGH reduced Zn^{2+}-, but not EGF-, induced EGFR phosphorylation (Figure 6B). At a

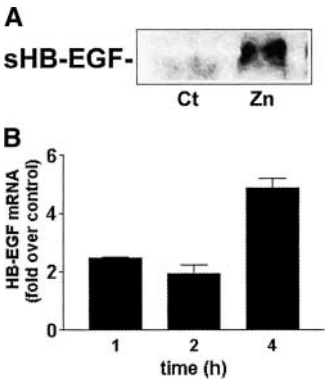

Figure 5. Zn^{2+} exposure resulted in HB-EGF release and mRNA overexpression in NHBE cells. NHBE cells grown to confluence were starved overnight. (*A*) Cells were pretreated with an EGFR antibody (LA1) and then exposed to medium control (Ct) or 100 μM Zn^{2+} for 2 h. Culture medium was collected and then precipitated with heparin-Sepharose beads overnight at 4°C. The precipitated beads were eluted with SDS gel sample and subjected to Western blotting and detected with goat anti-human HB-EGF antibodies. (*B*) Starved NHBE cells were stimulated with 100 μM Zn^{2+} for 1, 2, and 4 h. Total RNA was extracted and reverse transcribed. Quantitative PCR was performed using the TaqMan method. HB-EGF mRNA abundance was normalized to the abundance of GAPDH mRNA. The HB-EGF mRNA level was shown as fold increase over control.

similar concentration, the inhibitor of MMP-2 or MMP-9 did not show similar inhibition of Zn^{2+}-induced EGFR phosphorylation (data not shown). Given that Zn^{2+}-induced EGFR phosphorylation was mediated by released HB-EGF, the effect of NNGH on Zn^{2+}-induced HB-EGF release was evaluated. NHBE cells were incubated with 40 μM NNGH for 30 min before treatment with 100 μM Zn^{2+} for 2 h. Immunoprecipitation and blotting of HB-EGF released into the medium showed that NNGH could inhibit HB-EGF release from NHBE cells exposed to Zn^{2+} (Figure 6C). These data suggested that MMP-3 might be somehow involved in Zn^{2+}-induced EGFR phosphorylation, mediated by HB-EGF shedding.

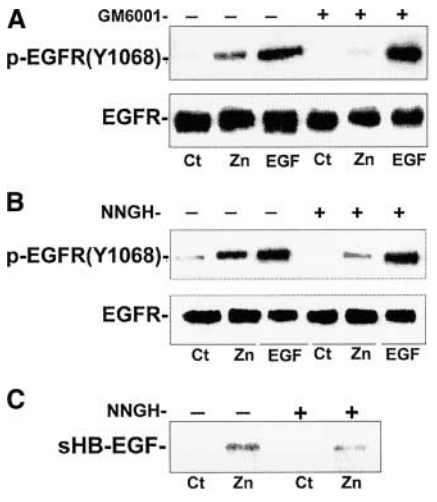

Figure 6. MMP inhibitor suppressed Zn^{2+}-induced EGFR phosphorylation as well as HB-EGF release. Confluent NHBE cells were starved and pretreated with an MMP inhibitor GM6001 (40 μM, 30 min), or an MMP-3 inhibitor NNGH (40 μM, 30 min) before treatment with medium control (Ct) or 100 μM Zn^{2+} for 2 h. Phosphorylated EGFR (*A*, *B*) was measured as described above. (*C*) NHBE cells were pretreated with 40 μM NNGH for 30 min. Released HB-EGF was trapped and detected in the medium from cells untreated or treated with 100 μM Zn^{2+}. Data shown are representative of three separate experiments.

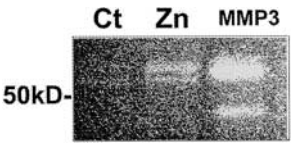

Figure 7. Zn^{2+} exposure increased the release of MMP-3 from NHBE cells. Confluent NHBE cells were starved before being stimulated with medium control (Ct) or 100 μM Zn^{2+} for 2 h. Cell medium was collected and precleared. MMP-3 proteins were precipitated with a mouse anti-human MMP-3 antibody overnight at 4°C. Precipitated MMP-3 was subjected to nonreducing prestained casein gel. Released MMP-3 was visualized as a clear band against a dark blue background. Purified human MMP-3 (both latent and active forms presented) was used as a positive control.

Considering that most of the MMP inhibitors exert their effect through interaction with a zinc atom in the catalytic domain of MMP (32), the specificity of NNGH was evaluated by measuring Src phosphorylation at tyrosine 416 induced by Zn^{2+} as described before (16) in NHBE cells. It was found that 50 μM TPEN (a known zinc chelator) significantly blocked Zn^{2+}-induced Src phosphorylation but 40 μM NNGH showed an only minimal effect (data not shown), implying that the inhibitory effect of NNGH on Zn^{2+}-induced EGFR phosphorylation was not mainly dependent on chelation of exogenous zinc ions.

Zn^{2+} Exposure Increases the Release of MMP-3 from NHBE Cells

The above data suggested that MMP-3 might participate in Zn^{2+}-induced EGFR phosphorylation. We therefore immunoprecipitated MMP-3 from cell culture media of Zn^{2+}-exposed NHBE cells using a monoclonal antibody and evaluated its functional capacity using casein zymography. NHBE cells were treated with 100 μM Zn^{2+} for 2 h. The culture medium was then collected and MMP-3 protein was precipitated with a mouse anti-human MMP-3 antibody. Precipitated MMP-3 was resolved on a 4–16% precast casein gel. Purified human MMP-3 (both latent and active forms presented) was used as a positive control. As shown in Figure 7, two main MMP-3 forms (a 58 pro-form and a 45 kD active form) were seen in the sample of positive control. There were pro-forms of MMP-3 existed in the medium of Zn^{2+}-exposed cells (Figure 7). We failed to detect any active MMP-3 forms released from Zn^{2+}-exposed cells. In comparison, only minimal MMP-3 appeared in the medium of untreated cells. Thus, Zn^{2+} exposure caused an increased release of MMP-3 from NHBE cells.

Discussion

Cross-communication between diverse signaling systems is well recognized in the cellular stimulus–response conversion networks and allows the translation of complex environmental conditions into appropriate reactions and adaptations (9). Recent studies have demonstrated that the EGFR acts as a key element for signal integration and is "transactivated"

by a multitude of stimuli (33). The involvement of cell surface receptors (34), nonreceptor tyrosine kinases (35), the serine/threonine kinase PKC (26), and metalloproteinases (36) has been investigated in transactivating EGFR. The EGFR transactivation mechanism is subjected to different cell type–characteristic regulatory influences (9). Our previous studies employing murine fibroblasts (16) and A431 cells (17) showed that Zn^{2+} exposure may induce rapid EGFR transactivation through an Src kinase–dependent mechanism. In contrast to previous observations, the current study demonstrated that exposure of NHBE cells to a lower concentration of zinc ions resulted in EGFR phosphorylation through an alternative mechanism involving the release of receptor ligands, which may be mediated by matrix metalloproteinases (MMPs).

Ectodomain shedding and release of soluble growth factors from the cell surface represents an important and efficient strategy to regulate the activity of transmembrane proteins such as EGFR (37). However, the specificity and extent to which these growth factors are involved in EGFR activation may vary with stimuli and cell type. For example, thrombin-induced EGFR transactivation is dependent on HB-EGF in vascular smooth muscle cells (38). Carbachol-induced EGFR transactivation requires extracellular release of TGF-α in T84 cells (27). Interferon γ triggered EGFR signaling through HB-EGF, TGF-α, and amphiregulin in human bronchial epithelial cells (19). In our study, the suggestion that HB-EGF might act as a major activator of EGFR phosphorylation in NHBE cells exposed to Zn^{2+} is supported by a number of observations. First, the EGFR antibody against the extracellular ligand–binding domain blocked Zn^{2+}-induced EGFR phosphorylation; second, an HB-EGF–neutralizing antibody ablated Zn^{2+}- but not EGF-induced EGFR phosphorylation; and third, the released HB-EGF was detected in the medium of Zn^{2+}-treated cells. Moreover, exogenous administration of recombinant human HB-EGF increased EGFR phosphorylation in these cells. In addition, of several known EGFR ligands, only HB-EGF mRNA expression was elevated following Zn^{2+} treatment. It should be noted that TGF-α may also participate in Zn^{2+}-induced EGFR, because a TGF-α–neutralizing antibody could inhibit Zn-induced EGFR phosphorylation to some extent. However, EGF cleavage was not required for Zn^{2+}-induced EGFR phosphorylation.

HB-EGF is synthesized as a membrane-anchored precursor protein of 208 amino acids that is subsequently cleaved on the cell surface to yield a soluble growth factor of 75–86 amino acids (39). Search for the proteolytic enzymes responsible for HB-EGF cleavage and subsequent EGFR transactivation has led to the study of metalloproteinases (36, 40). Metalloproteinases are a large family of zinc-dependent endopeptidases, which includes subfamilies such as the MMP and the ADAM (a disintegrin and metalloproteinase) families (41). Recent studies have shown that HB-EGF shedding could be processed through ADAM such as ADAM9 (42, 43), ADAM10 (44), ADAM12 (45, 46), tumor necrosis factor-α–converting enzyme (TACE)/ADAM17 (47), or MMP such as MMP-3 (48), -7 (49), -2, and -9 (50, 51). Apparently, HB-EGF shedding can be regulated by diverse metalloproteinases in different exposure contexts. In our experimental system (primary NHBE cells), MMP-3 appeared to mediate Zn^{2+}-induced HB-EGF release, further leading to EGFR phosphorylation although the active forms of MMP-3 were not detected in our assay. Only Zn^{2+} exposure induced MMP-3 release. The specific MMP-3 activity inhibitor, NNGH, blocked Zn-induced HB-EGF shedding and also suppressed the subsequent EGFR phosphorylation. Although the inhibitors of MMP-2 and -9 did not exert an inhibitory effect on Zn-induced EGFR phosphorylation, the involvement of other MMPs in this process cannot be excluded because the MMP-3 inhibitor did not completely inhibit Zn-induced HB-EGF shedding.

MMP-3 is a "soluble" or non–membrane-bound zymogen secreted as a proenzyme that can be activated through the disruption of a Cys-Zn^{2+} (cysteine switch) interaction, and the ensuing removal of its amino-terminal prodomain (52, 53). Regulation of MMP is exerted at many levels, involves both transcriptional and post-transcriptional mechanisms, and appears to be highly cell- and tissue-specific (53). The mechanism regulating MMP-3 in our system remains elusive. We were unable to detect direct activation of MMP-3 by zinc ions (data not shown). Src kinase activity was recently reported to participate in estradiol-induced MMP-2/MMP-9 activation in cultured MCF-7 cells, leading to HB-EGF–mediated EGFR transactivation (50). However, we failed to inhibit the Zn^{2+}-induced release of MMP-3 using a specific Src kinase inhibitor (PP2) although Src kinase activity was needed for Zn^{2+}-induced EGFR phosphorylation. Whether Src kinase triggers the EGFR phosphorylation through other MMPs will be examined.

It has been noted that Zn^{2+} concentrations in certain biological compartments can reach as high as 300 μM (54). However, Zn^{2+} uptake and its role in intracellular communication is poorly understood. The signaling mechanisms activated in response to Zn^{2+} exposure have been the subject of a number of recent studies (55). A study showed the existence of a putative Zn^{2+}-sensing receptor in colonocytes (56). The concentration of atmospheric Zn^{2+} to which NHBE would be expected to be exposed *in vivo* is difficult to estimate, as such calculations are dependent on a number of variables, such as rates of deposition, particle solubility, and clearance, which in turn are determined by physicochemical and physiologic parameters that can vary significantly. The concentrations of Zn^{2+} used in this study are intended to test the potential response to exposure to noncytotoxic exposures that may occur in the environment.

EGFR phosphorylation and related downstream signaling was shown to play an important role in Zn^{2+}-containing ambient particulate matter-induced expression of proinflammatory mediators in NHBE cells (5). Moreover, in other experiments employing the same Zn^{2+} exposure conditions as used in this study, exposure of NHBE cells to 100 μM Zn^{2+} significantly increased the expression of the proinflammatory protein cyclooxygenase-2 (COX-2), and this effect was ablated by the specific EGFR kinase inhibitor PD153035 (W. Wu, unpublished data). Comparing the findings of the present study to our previous studies (16, 17) in which a shorted exposure to a higher concentration of Zn^{2+} was used, it is evident that multiple concentration-dependent mechanisms of signaling may be initiated by

Zn^{2+} exposure. Preliminary studies in our laboratory suggest that the mechanism activated in response to Zn^{2+} exposure is dependent on the rate of Zn^{2+} influx into the cell, which is a function of the permeability of the cell to divalent metal ions such as Zn^{2+} (J. M. Samet and W. Wu, unpublished observations). These observations may have implications relevant to the susceptibility of lung cells to exposure to ambient concentrations of Zn^{2+} in certain disease states in which cell permeability may be compromised.

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